

NASA
Final Technical Report for NAG8-1590

December, 2004

**In-situ Optical Waveguides for Monitoring and Modifying
Protein Crystal Growth**

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Abstract:

The use of electric fields in the growth of protein crystals was investigated, both theoretically and experimentally. We used dc, ac and optical fields to change the spatial distribution of proteins. Dc fields had only local effects, due to the conductivity of the growth solution. We found that for low frequency fields, movement of the buffer and salt ions dominated, and that for high frequency ac fields, dielectrophoretic effects could be useful for relocating growing protein crystals. The most promising result was that for optical fields, a large gradient in the field could be used to capture a crystal, and observe growth in-situ. This concept could be developed into an experimental setup compatible with automated x-ray diffraction measurements in microgravity.

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Introduction

Fundamental knowledge of protein structure is of primary importance for biochemical studies, due to the link between form and function in these materials. Crystallization is typically a key step, due to the use of x-ray diffraction to yield high-quality information about the precise three-dimensional location of the amino acids in folded proteins. In the search for ways to improve crystallization, and to make it possible to localize and preferentially promote growth of a crystal, there has been recent interest in the use of electric, and magnetic fields to influence these factors.

Surprisingly little appears in the literature about the use of local electric fields to modify protein crystal nucleation and growth. There was an early suggestion [Tiller] that fields might be used to influence growth, and one modified electrophoresis investigation where protein crystallization was enhanced [Chin, et al.]. In the electrophoresis experiment, a dialysis membrane was placed upstream of one of the electrodes, and protein collected on this membrane. The concentration enhancement, and possible dipole alignment were suggested as the mechanisms responsible for the growth of crystals on the membrane. There is also a pair of theoretical papers exploring the relationship between protein alignment, shear-induced flow, and the presence of an electric field [Noever]. Noever suggests that moderate fields (on the order of hundreds of Volts/cm) should suppress shear-induced flow of proteins near a surface, and enhance alignment between proteins.

Some recent studies have examined the ability of electric or magnetic fields to manipulate the quality, orientation, and speed of crystal growth [Taleb, Naney, Ataka, Sato, Mirkin]. However, none have intensely studied effects on crystal location. This research program focused on controlling where a protein crystallizes with the application of an electric field.

The primary approaches that could be taken to force this localization are: increasing the concentration of protein at a particular location to make nucleation there more likely, increasing the supersaturation ratio at a particular location through controlling the concentration of precipitant or the local temperature, or placing a seed crystal at a particular location. Our investigation has spanned this range, with the exception of temperature control, using electric fields to move around the protein and other charged species.

Objectives, as stated in the original proposal:

To establish the use of electric field enhanced nucleation and growth of protein crystals
To reduce gravitationally induced convection using fields
To develop optical methods for imposing local surface fields and monitoring protein alignment and attachment

Modifications : we added establishment of baseline expertise on the growth of lysozyme crystals, and moved from waveguide optical fields to free-space propagation.

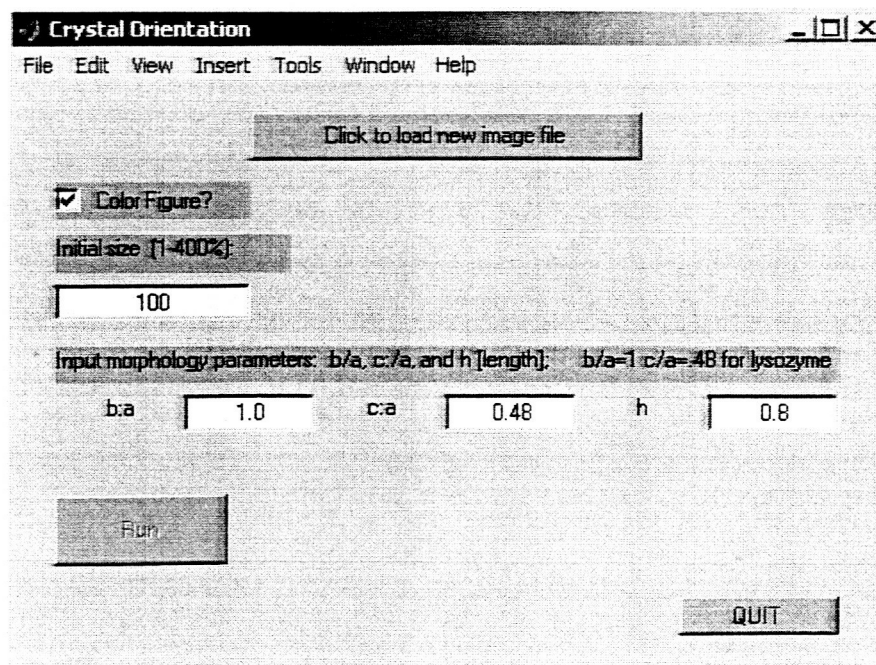
Results/Accomplishments

Although we did not ultimately use optical fibers for the research, we accomplished almost all of the original objectives of the proposal. Theoretical results included a Monte Carlo simulation of protein aggregation in the presence of an electric field, and modeling of dielectrophoresis of proteins in solution. In addition, we developed a computer program that allows the determination of crystal orientation from a microscope image, which is available as freeware to the community. Experimentally, we were able to demonstrate growth modification for protein crystals in a dc field, use evanescent optical fields to detect the increased concentration of proteins at a surface under the influence of an electric field, and demonstrate optical trapping and monitoring of the growth of protein crystals. These results are described more thoroughly in the pages that follow, including material from the theses and publications funded by the grant.

Theory, modeling

Determination of crystal alignment

There have been a few papers on lysozyme crystal growth in the presence of an electric field, with enhanced growth or orientation reported. One of the first experimental areas that we undertook was to see if these results were reproducible. In order to analyze the data, which is in the form of micrographs taken parallel to the direction of the applied field, in a quantitative manner, we developed a computer code to determine the crystal alignment. The program yields a value for the angle between the c-axis and the applied field. It uses open-source MATLAB code, and presents a graphical user interface for aligning a skeleton cage with the image in the micrograph. When the user aligns the cage with the image, the angle between the relevant directions can be read from the screen. Below, we show the initial screen presented to the user:



First screen of the alignment program, for data input.

Once the user selects a file for data analysis, and chooses the parameter that controls the aspect ratio of the crystals in the tetragonal form, the computer loads the image, and draws a skeleton cage in the default position, as shown below.

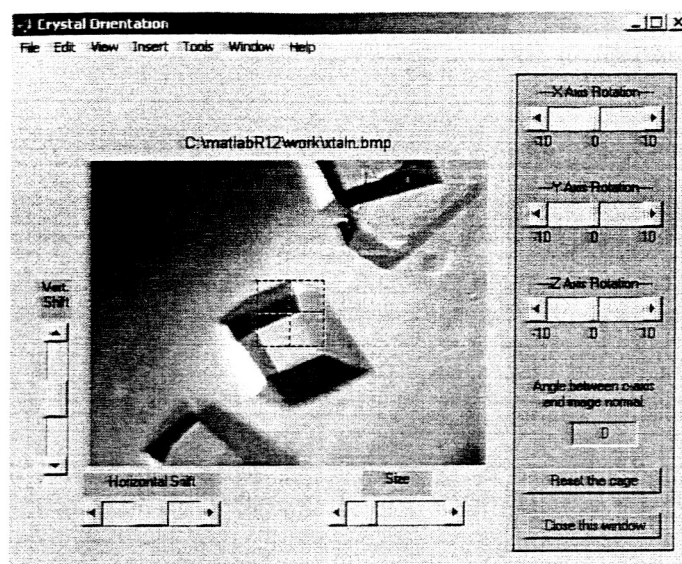
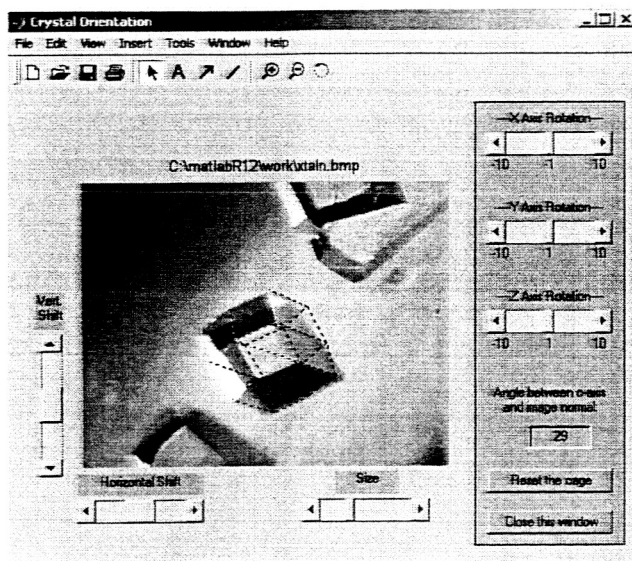


Image screen of the alignment program, showing GUI slider controls for rotation, shift and size of the cage.

The user then interactively adjusts the position, size and orientation of the cage until the edges of the crystal image all line up with the edges of the cage. The results of user manipulation of the cage, for a truncated crystal, are shown below. The angle between the c-axis and the image normal (29 degrees) is output to an on-screen window.



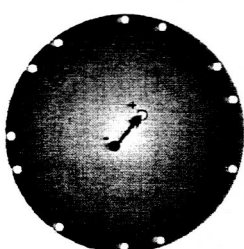
Screen shot after cage alignment

Use of this program by several individuals yielded an accuracy of 2-3 degrees with a average manipulation time of one to two minutes. Recently, this software has been used to analyze the growth rate of HEWL crystals trapped in optical tweezers. This program is available to the public on the web at:

<http://www.dartmouth.edu/~ujg/downloads/matlab.html> .

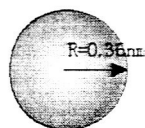
Monte Carlo simulation of protein aggregation in electric fields

Modeling of either protein folding or protein crystallization is a daunting computation task, which is slowly yielding to the efforts of large groups interested in this area. Our task was somewhat less ambitious – using a simple model of charged bodies moving in a fluid, to look for dielectrophoretic effects and clustering due to the presence of either a precipitant, or an applied electric field. We undertook a model using finite boundary conditions, large spheres with uneven charge distribution as protein molecules, and small spheres of uniform charge as the salt ions. The H^+ and OH^- ions were not treated explicitly in the model, but an effective radius for the hydrated ions was assumed.



○ Positive charge
● Negative charge

Model of protein molecule



Na^+ (hydrated)



Cl^- (hydrated)

Model radii of hydrated sodium and chlorine ions

Within the bounds of this simple model, we were able to look at the effects of pH and applied field on a very small sample of the solution. The small values of the Debye screening length for solutions of realistic salinity make this a surprisingly acceptable limitation. In addition to single particle interactions, we considered the effect of aggregated charge clusters, with their associated dipole moments.

The forces on protein molecules include the electrostatic forces and the osmotic force exerted by the surrounding salt ions. The driving forces on a protein molecule are:

$$F_i = F_{qq} + F_{dq} + F_{dd} + F_{osm} \quad (1.1)$$

Where the first term is the Coulomb interaction for spherically symmetric distributions (no torques), F_{dq} is the dipole- charge term, F_{dd} is the dipole term, and F_{osm} is the osmotic force on the protein, due to concentration gradients induced in the salt ions in solution. These terms were evaluated as follows:

Salt ions and the charges on the surrounding protein molecules all exert electrostatic forces on each charge that a protein molecule carries. This force can be expressed as:

$$F_{qq} = \sum_i \left(\sum_j \frac{Q_i \cdot Q_{Na^+}}{4\pi\epsilon_0\epsilon_r r_{ij}^2} + \sum_k \frac{Q_i \cdot Q_{Cl^-}}{4\pi\epsilon_0\epsilon_r r_{ik}^2} + \sum_l \sum_m^{protein\ charge} \frac{Q_i \cdot Q_{lm}}{4\pi\epsilon_0\epsilon_r r_{im}^2} \right) \quad (1.2)$$

where j indicates the jth Na⁺ ion and k indicates the kth Cl⁻ ion in the solution and i is the ith charge on a protein molecule. Q_{lm} is the mth charge on the protein molecule l. Charges that protein carries can be positive or negative. The charge distribution on the surface used here is approximately uniform, with the dipole moment of the protein introduced explicitly. F_{dq} is the force between dipole moments and charged particles.

$$F_{dq} = F_{Na^+} + F_{Cl^-} + F_p$$

$$= \sum_i^{Na^+} \frac{Q_i}{4\pi\epsilon_0\epsilon_r} \left(\frac{Q_p}{r_{pi}^2} + \frac{Q_n}{r_{ni}^2} \right) + \sum_j^{Cl^-} \frac{Q_j}{4\pi\epsilon_0\epsilon_r} \left(\frac{Q_p}{r_{pj}^2} + \frac{Q_n}{r_{nj}^2} \right) + \sum_k^{ProteinCharge} \sum_m \frac{Q_m}{4\pi\epsilon_0\epsilon_r} \left(\frac{Q_p}{r_{pm}^2} + \frac{Q_n}{r_{nm}^2} \right) \quad (1.3)$$

Positive and negative salt ions as well as the charges on the surrounding protein molecules impose such a force on the effective dipole moment of each protein molecule, as shown in equation (1.3), where Q_p and Q_n are the charges at the positive end and the negative end of the effective dipole moment of each protein molecule and Q_m is the mth charge on the protein molecule k.

There are also dipole-dipole interactions among protein molecules. Each protein molecule has a dipole moment induced by the local electric field. There are contributions to this electric field from salt ions and the surrounding charged proteins. We assumed that the induced dipole moment follows equation (1.4):

$$\mu = Q \cdot d = Volume \alpha_0 E = \alpha E \quad (1.4)$$

where α_0 is the polarizability. Then F_{dd} is the expression for dipole-dipole term from local proteins:

$$F_{dd} = \sum_i^{local\ protein} \left[\frac{Q_p}{4\pi\epsilon_0\epsilon_r} \left(\frac{Q_{ip}}{r_{pp}^2} + \frac{Q_{in}}{r_{pn}^2} \right) + \frac{Q_n}{4\pi\epsilon_0\epsilon_r} \left(\frac{Q_{ip}}{r_{np}^2} + \frac{Q_{in}}{r_{nn}^2} \right) \right] \quad (1.5)$$

F_{osm} is the osmotic force imposed on the protein molecules by the surrounding salt ions, and is present whenever there is a local concentration gradient. Once the electrostatic force is screened out by the salt ions, the osmotic force would be a dominant factor in protein crystallization. According to the Van't Hoff equation which is for the osmotic pressure in gas and dilute solutions:

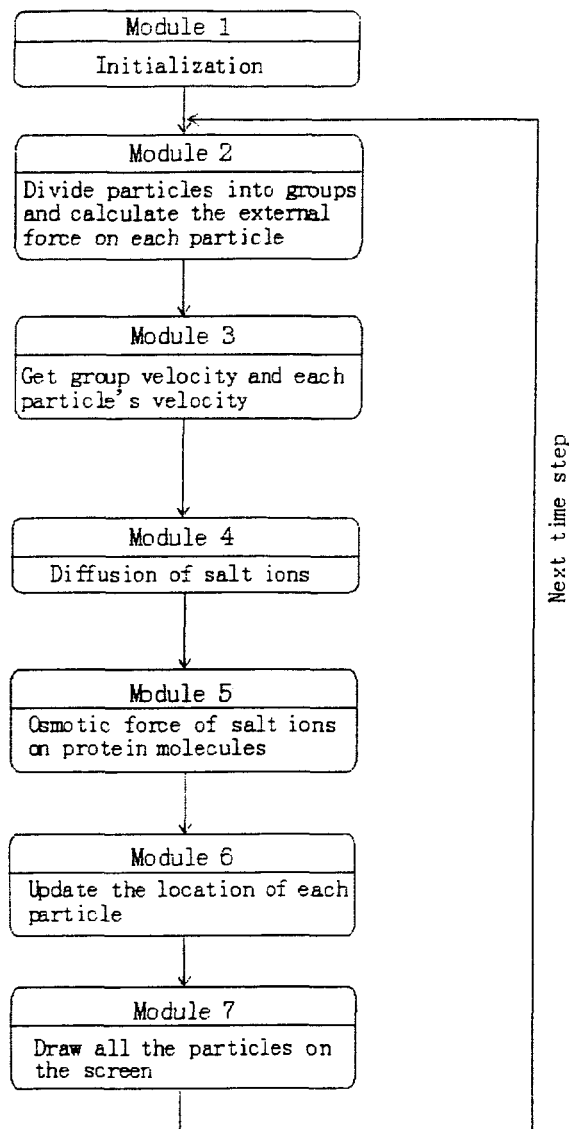
$$\Pi V \approx nRT \quad (1.5)$$

where Π is the osmotic pressure of the solution, proportional to the amount of solute n. The equation is valid for gas and dilute solution. For more concentrated solutions, there is a modification:

$$\Pi V \approx nRT (1 + Bn) \quad (1.6)$$

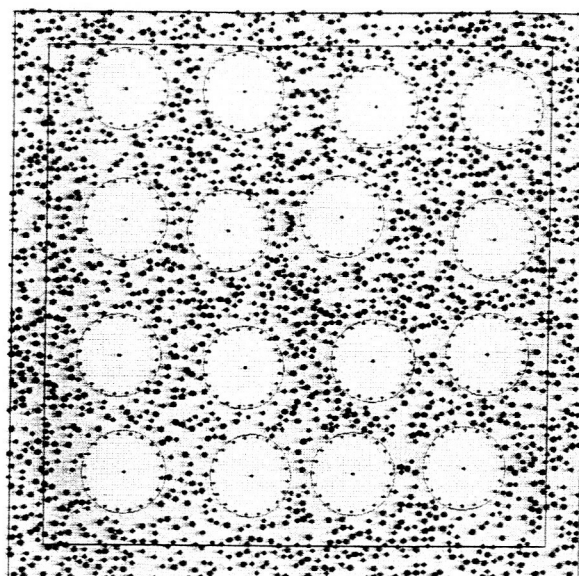
where B is the osmotic viral coefficient. However, in the current problem the effect of this correction is small. Similar equations were used for the forces on the salt ions.

Below, we show the flow of the simulation program, which was written in C++.



Flowchart for the simulation code

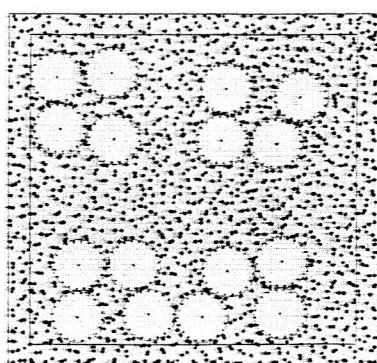
The initial condition was set by randomly assigning non-overlapping locations for the salt ions and protein molecules, and the calculations are performed in two dimensions. A sample plot of the initial conditions is shown below.



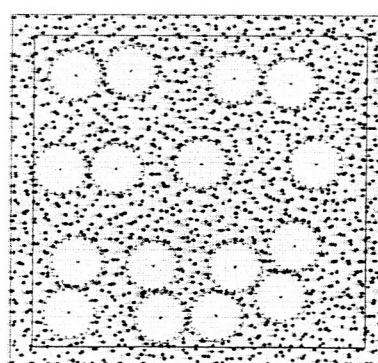
Initial conditions for studies of protein aggregation

The large yellow circles are the protein molecules, and the small red and blue spheres are the sodium and chlorine ions, respectively. The surface charge on the proteins varies with the pH of the solution. The protein concentration was the equivalent of approximately 20mg/ml.

The output of the program is graphical, showing aggregation and/or displacement. In order to derive a quantitative measure of solubility, K is defined as the ratio of the numbers of protein molecule groups to the size of the maximum group. For example, $K = 3/8$ in Fig.1-1(a) and $K = 7/6$ in Fig.1-1(b). K can be regarded as an indicator of the solubility. Larger K value implies larger solubility. K in Fig.1-1 (a) is smaller than in Fig.1-1(b), which means the protein in Fig.1-1(a) is easier to aggregate.



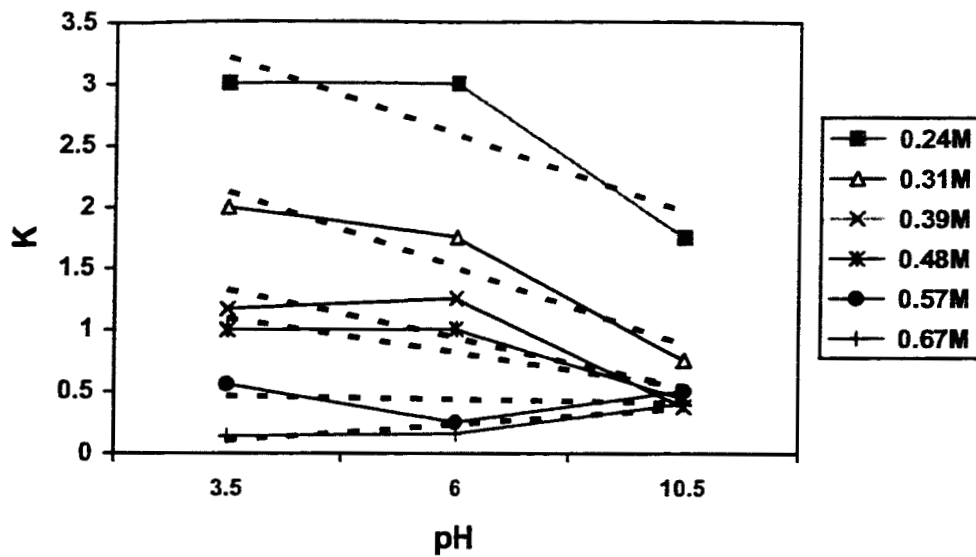
(a) Net charge=2



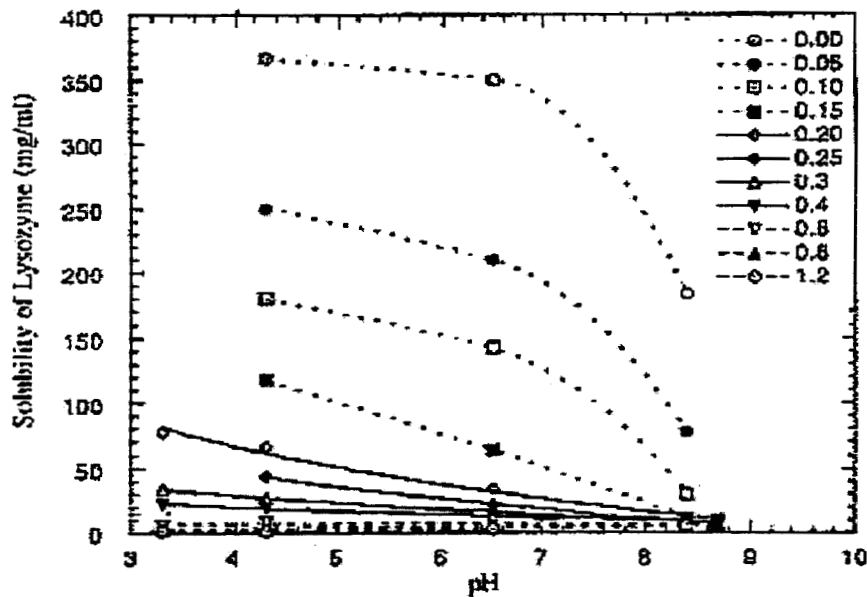
(b) Net charge=6

Protein molecule distribution after 3000 sec with NaCl concentration equivalent to 0.4M

Using this parameter, we were able to measure the calculated and experimental solubility of lysozyme, as shown below. The overall shape and NaCl concentration dependence of the curves is consistent.



K as a function of pH and NaCl concentrations



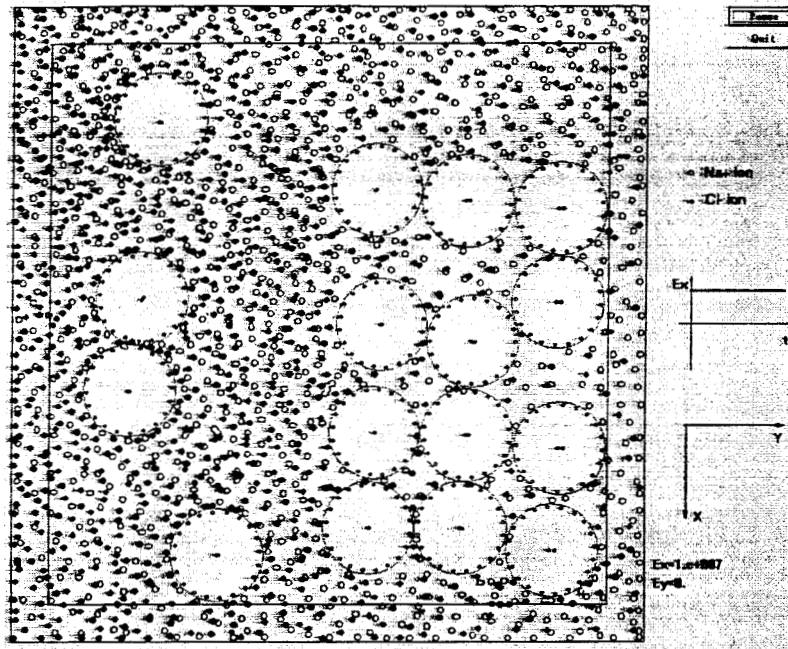
Solubility of lysozyme as function of pH, at 18°C and various NaCl concentrations (Ries-Kautt)

Applying an external field

Strong electric fields have been applied to lysozyme solutions resulting in large differences between with and without the external field. In this program, external electric fields are also simulated.

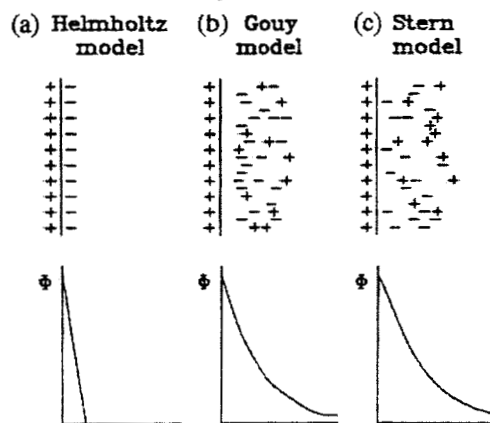
The simulated uniform field in the program is along the positive X direction. First we tried a field of intensity $E_x = 1 \times 10^7$ V/m. This is an extreme which can not be achieved in

the lab. The voltage range of the external field used in the experiments is from 10^{+5} to 10^{+6} V/m. For 10^{+7} V/m, the effect of the external field is obvious. As shown in Fig.3-4, there are much more positive Na^+ ions (white spots in the figure) at the right side of the figure and more Cl^- ions (dark spots) at the left side of the figure.



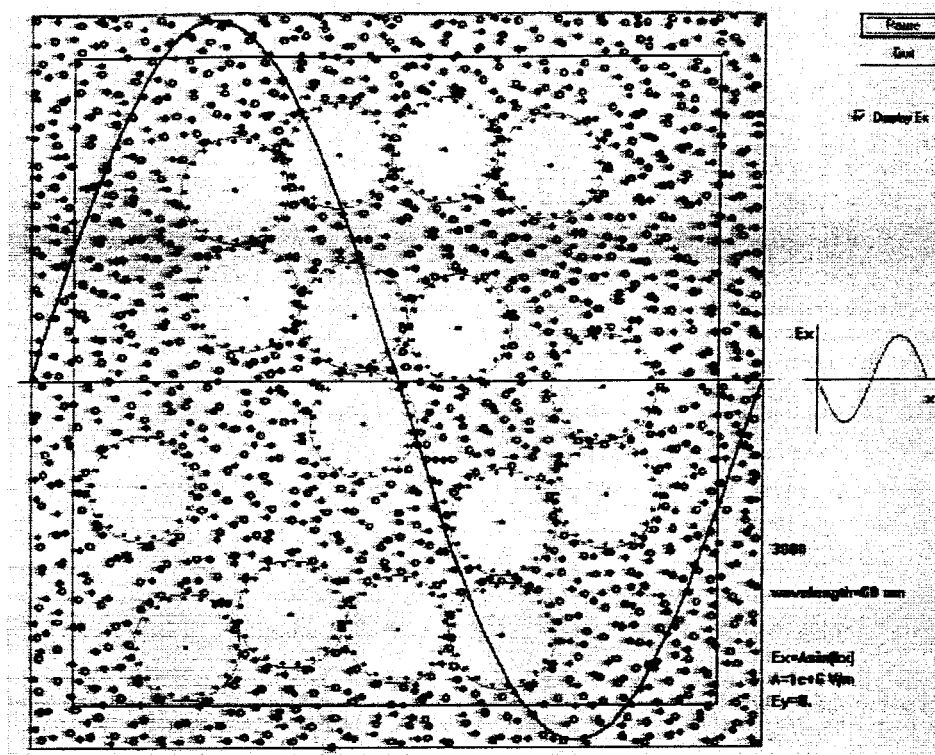
The lysozyme solution under a uniform external field of 10^{+7} V/m

This is reasonable since the positive ions move along the direction of the external field and the negative ions move in the opposite direction. We can also compare this with the three models of the electrical double layer: Helmholtz model, Gouy-Chapman model and Stern model, which predict a surface layer with altered concentrations of the ions.



For more realistic field strengths, we found that the amount of aggregation in the bulk of the sample was not statistically affected by the application of a dc field. However, we found that the application of a non-uniform ac field, and the accompanying

dielectrophoretic forces looked quite promising. The results of application of an ac field are shown below:



Result of non-uniform field E_x after 3000 seconds

Based on this microscopic simulation result, we examined existing theories of dielectrophoresis, as they apply to lysozyme.

Dielectrophoresis modeling of lysozyme in solution

Another approach to localizing growth of protein crystals is to manipulate the tetramers, octamers, critical nuclei, or small seeds that form early in the crystallization process. This can be achieved by the use of non-uniform ac fields, since any neutral polarizable entity will be driven to the high field region in the presence of a gradient field, throughout the phases of each cycle. The dielectrophoretic (DEP) force on a particle suspended in a medium, in an ac field E , is

$$F_{DEP} = \frac{3}{2} Vol \cdot \text{Re}(f_{CM}) \cdot \nabla |E|^2 \quad (\text{Pohl}) \quad [2.1]$$

Where Vol = particle volume, and f_{CM} is a size and dielectric function dependent quantity.

For cylindrical electrode symmetry, this force is always radial. From Eqn. 2.1, it is evident that a relationship exists between the particle size, field strength, and Clausius-Mossotti factor. By taking into account the thermal motion which must be overcome in order for a particle to be successfully moved by DEP, the following condition results:

$$a^3 \geq \frac{kT}{40\pi E^2 \epsilon_m \text{Re}(f_{CM})} \quad [2.2]$$

Where a = particle radius

The direction of motion expected for a particle in any given medium can be determined by the polarizability factor, f_{CM} , where:

$$f_{CM} = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2\epsilon_m^*} \quad [2.3]$$

ϵ_p^* = complex permittivity of particle

ϵ_m^* = complex permittivity of suspending medium

$$\epsilon^* = \epsilon - \frac{i\sigma}{\omega} \quad [2.4]$$

ϵ = permittivity

σ = conductivity

ω = angular frequency of applied field

From equations 2.1-2.4, the necessary field, frequency, and solution parameters can be found for the dielectrophoresis of any neutral, polarizable body.

Previously, DEP has been used in the separation of mixtures of biomolecules. Huang et al predicted the frequency response, based on the Clausius-Mossotti factor, for bacteria, yeast, and two proteins. They successfully separated bacteria from yeast, and proteins from whole blood samples, with the combined application of DC and AC fields. The experimental frequencies ranged from 10 to 200 KHz with suspending medium conductivities of 20 to 1200 $\mu\text{S}/\text{cm}$ (2001). Recently, DEP has been widely used to manipulate colloidal spheres in solution [Docoslis 2002, Schnelle 2000, Bakewell 2004].

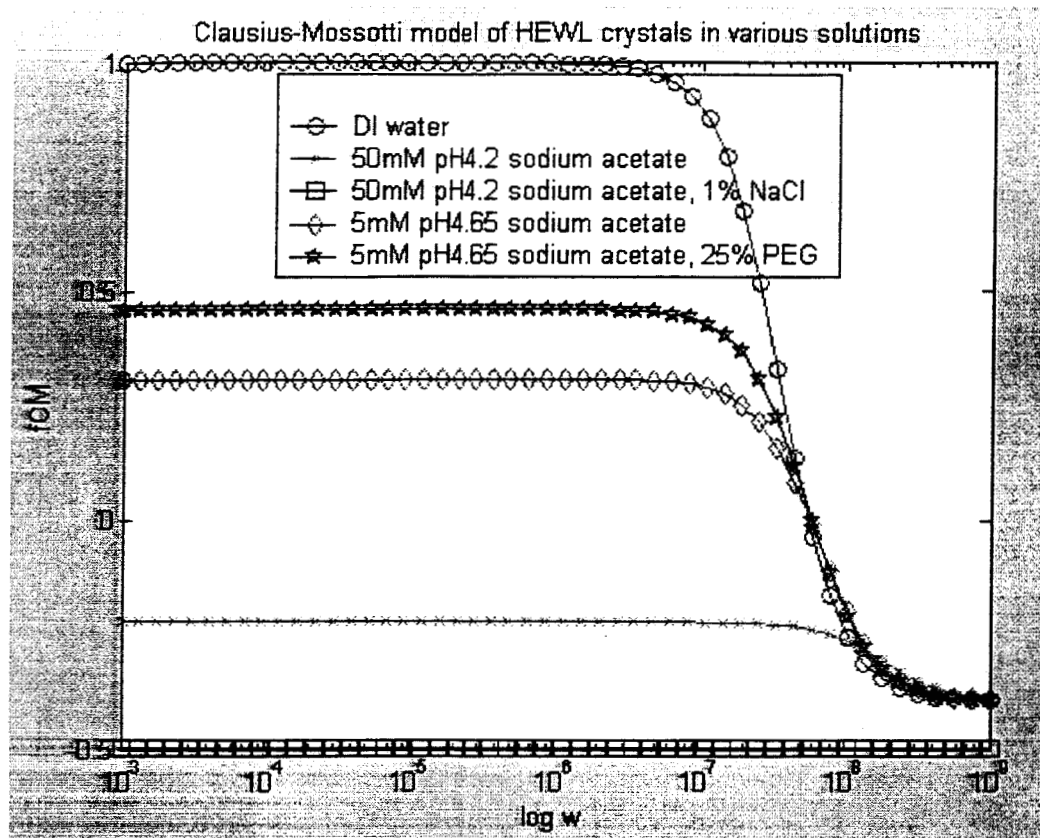
Under the right conditions, it should be possible to drive a neutral HEWL crystal to the location of a point electrode with positive DEP. A relatively safe estimate of the diameter of a hen egg white lysozyme (HEWL) crystal is $\geq 1.0\mu\text{m}$. As is evident from Eqns 2.2 and 2.3, it is necessary for ϵ_p^* and ϵ_m^* to be quite different in order to move a

protein crystal that small with a reasonable applied voltage. Water has a relatively high dielectric constant (~ 80 up to 40 GHz), and is a major constituent of both protein crystals and buffer solution. At first glance it may seem that K_1 and K_2 must be close to that of water, with a difference small enough that it would be difficult to move the protein crystal. However, as shown by Elgersma et al, the dielectric constant of water bound in a protein crystal (30-40% in HEWL) is lower than that of free water.

At this time, the dielectric constant of a tetragonal HEWL crystal is not well known. There are several studies which have determined a range of values for HEWL in solution. Garcia-Moreno et al experimentally determined this value to be 28, which includes shielding of the interior of the lysozyme. Pitera et al used a simulation to arrive at the value of 25.7 for HEWL in solution, and 12.5 for a HEWL crystal unit cell (no suspending medium included in simulation). Morozova et al were able to isolate a whole, tetragonal HEWL crystal between the electrodes of a conductivity meter, in order to take measurements. They tested crystals that were grown in a 5mM complex buffer of pH 4.0, with 0.4% and 5.3% NaCl, to find conductivities of $157 \cdot 10^{-6}$ and $534 \cdot 10^{-6}$ S/cm, respectively. The variation of crystal conductivity based on salt concentration during growth is most likely a result of Cl^- ions being incorporated into the crystal lattice. It has been observed by others [Elgersma] that Cl^- ions are indeed part of a HEWL crystal, and should be taken into consideration. The figure below presents a model of the Clausius-Mossotti factor over these ranges of ϵ_p and σ_p to explore the expected behavior of seed crystals of HEWL in an electric field. The table shows permittivity and conductivity values used in the model.

	HEWL crystal ^d	DI H_2O ^e	50mM buffer ^a	50mM buffer ^a , 2% NaCl	5mM buffer ^b	5mM buffer ^b , 25% PEG ^c
Relative permittivity (ϵ/ϵ_0)	12.5	80	80	80	80	80
Electrical conductivity ($\mu\text{S}/\text{cm}$)	534	0.6	1180	184000	230	151

Notes: a: sodium acetate pH 4.0; b: sodium acetate pH 4.65; c: Polyethylene Glycol of molecular weight 3350; d: Crystal grown in 5% NaCl, 5mM complex buffer pH 4.0 by Morozova et al, 1996; e: as dispensed in lab.



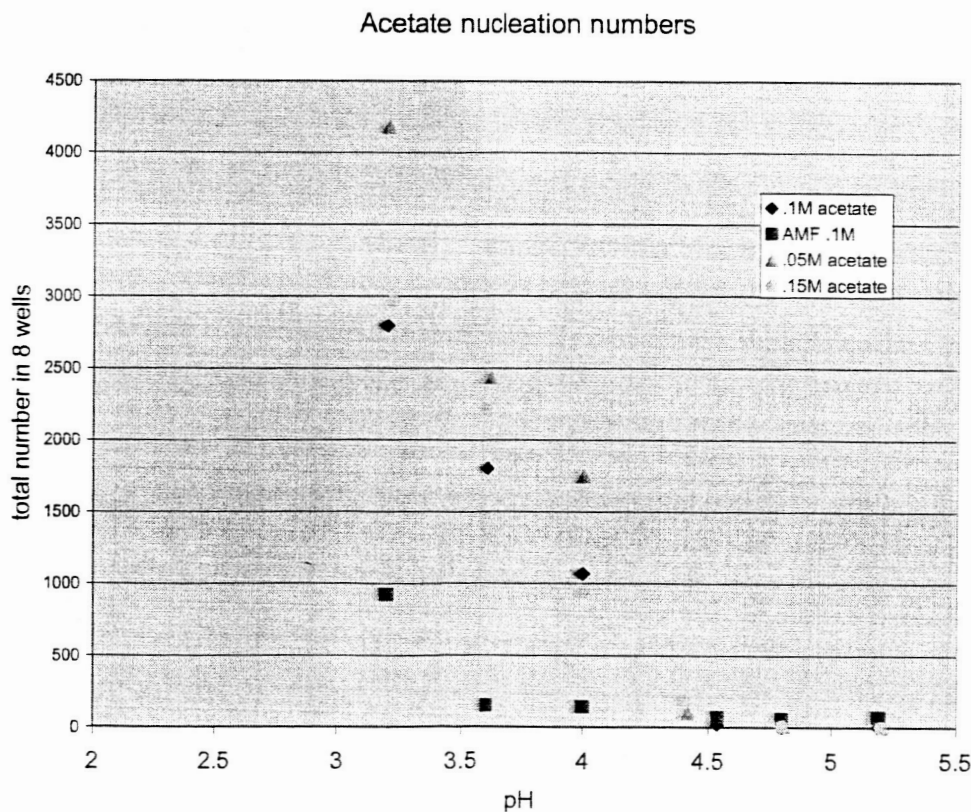
Values of the Clausius-Mosotti factor for HEWL

From the calculations, one would expect positive DEP of HEWL crystals in DI water and 5mM sodium acetate pH 4.65 (with and without polyethylene glycol added) at frequencies less than $\sim 10^8$ Hz, and negative DEP above that crossover frequency. For HEWL crystals in 50mM pH 4.2 sodium acetate (with and without NaCl), one would expect to observe negative DEP at all frequencies, based on the Clausius-Mossotti factor. The results of these calculations were used to guide our experimental investigations of dielectrophoresis of HEWL microcrystals.

Experimental results: nucleation and growth of lysozyme

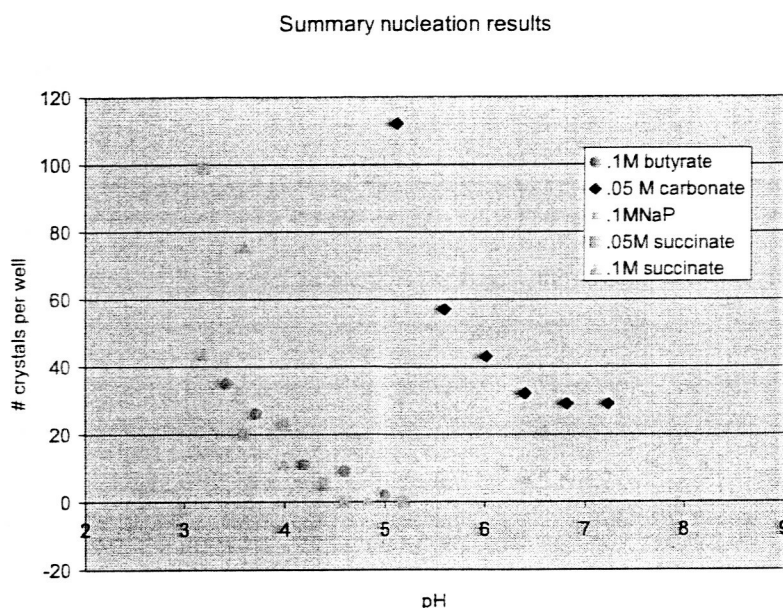
No field applied

We undertook baseline studies of the crystallization of lysozyme *without* applied fields, so that we would be able to control nucleation and growth of this protein as the electric field studies progressed. In particular, we studied the effect of pH, buffer choice, and buffer concentration on the nucleation of the crystals, and on their growth morphology. We discovered that the number of nuclei, under diffusive, rather than stirred, combination of precipitant and buffer was controlled effectively by the pH of the solution *relative to the pK_a* of the buffer being used. Prior results [Judge, et al] had indicated that in batch processing, there was a pH dependence, but in our work, we were able to correlate this to the pK_a of the particular buffer being chosen. This work is summarized in the graphs below. The first plot shows the results for acetate buffer, including results for several different acetate concentrations. In these studies, a mixture of buffered protein was added to a buffered salt solution at the indicated pH. The results are remarkably consistent between different buffer concentrations. Later results indicated that the actual numbers are different for the case where the protein is equilibrated first with the buffer, but that the overall result -- suppression of nucleation as $pH \rightarrow pK_a$ is quite general. The square data points are for lysozyme tagged with the fluorescent dye, AMF. The addition of this tag clearly interferes with the normal acetate-protein interactions.



*Numbers of crystals for an eight-well sample after 5 days of growth,
for acetate buffers*

The figure below shows our result for a range of buffers, and with the exception of the phosphate buffer, the trend is the same -- the nucleation rate is suppressed as the pK_a for the buffer is approached. The values of pK_a vary slightly with salt conditions, but approximate values for the buffers studied here are: acetate-4.73, butyrate-4.83, succinate-4.2 and 5.6, carbonate-6.34, and phosphate-2.12 and 7.2.

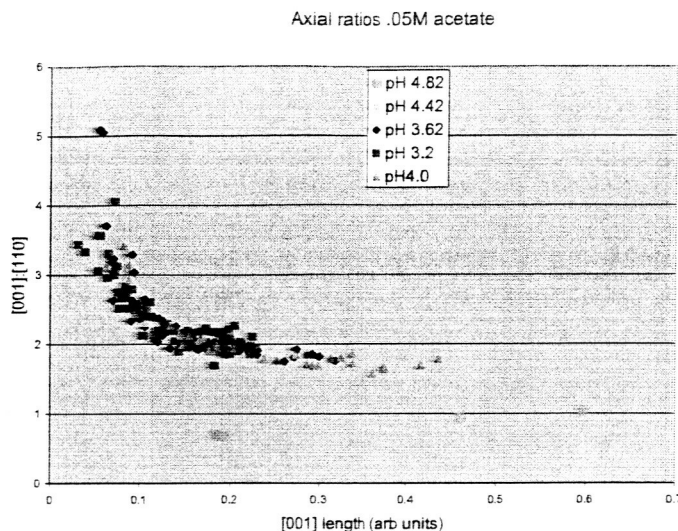


Number of crystals per well for a range of buffers

It is notable that the only buffer that shows little pH effect is the phosphate, where we are using pK_2 . In the succinate system, there is no indication that the number of crystals will rebound before the second titration point. It thus appears that the greatest suppression of nucleation is associated with the first ionization of the buffer.

The other property of the crystals that was measured in the formate and acetate buffers was the aspect ratio of the (110) face. The length of the face in the [001] direction was measured, and compared to the width of the (110) face in the [110] type directions. The magnification used for all measurements was held constant, so that comparisons could be made from one plate to another. Judge et.al⁴ reported that the aspect ratio decreased as a function of pH in the acetate buffer system. Their results were for batch prepared crystals, where the size distribution was very narrow. In our experiments, where the solutions are not actively mixed, and nucleation occurs more slowly during the vapor diffusion, we observed a range of crystal sizes within each well. We found that the axial ratio was largely independent of pH, and that it scaled very well with the size of the crystal. The pH during growth biases the crystal size, as can be seen below. In Judge's experiments, the number (and size) dependence on pH, and their

narrow size distributions resulted in their sampling a very small portion of the curve at each pH.



Proteins crystallized with dc applied voltages

In a review of the literature, there are claims made about alignment of proteins in dc fields under two conditions -- with and without current flow. The first case effectively applies a field solely at the surface, while the latter has a sustained field throughout the crystal.

Without current flow

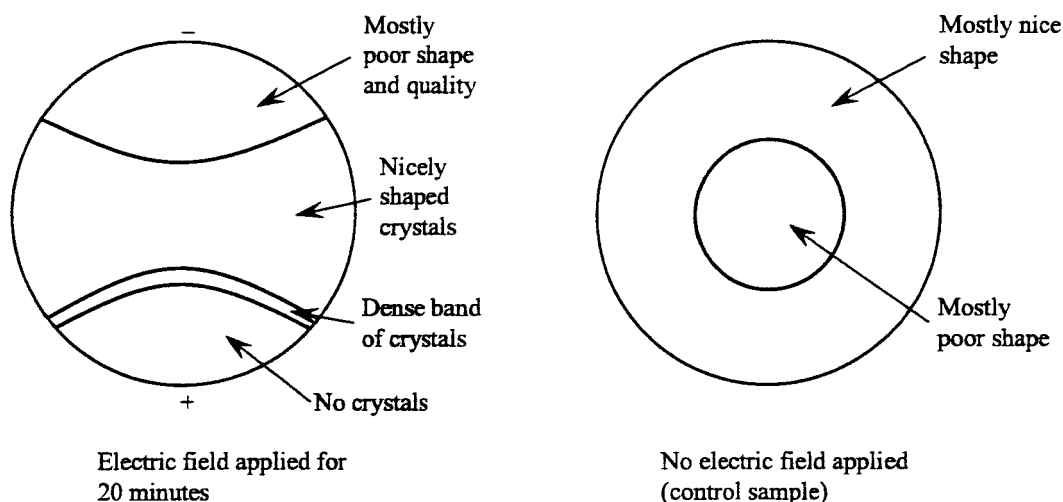
For the case of a protein crystal grown between insulating plates, the electrical equivalent is a pair of capacitors in series -- the conducting liquid in which the protein crystal grows contributes ions to the double layer described above. Under these conditions, unless nucleation occurs at the electrode surface, it is unlikely that orientation effects will be observed. Indeed, in our lab, where we did not perform the aggressive substrate preparation used by Nanev et al, we were able to use our software package (described above) to determine that there were only minor orientation effects in a number of different configurations. We attribute the disagreement with Nanev's work to the difference in surface preparation.

With current flow- electrophoretic growth

We also studied protein crystal growth after application of a true dc field, which for the conductivities of typical growth solutions, only occurs if there is a current flow sustained through the solution. The configuration that we used to study this was essentially a miniature electrophoresis gel, into which we loaded either natural or fluorescence-tagged lysozyme, so determine the effects of the field (and current) on the crystallization. Agarose gels with uniform, but low concentrations of lysozyme (8 mg/ml) were prepared, electrophoresis was performed at 20 V for 20 minutes (voltages

and times were chosen to mimic typical electrophoresis values, adjusted for our sample geometry). After electrophoresis, the gels were placed in the refrigerator for 10 days with a reservoir of 5% NaCl around them to promote crystallization by the vapor diffusion method.

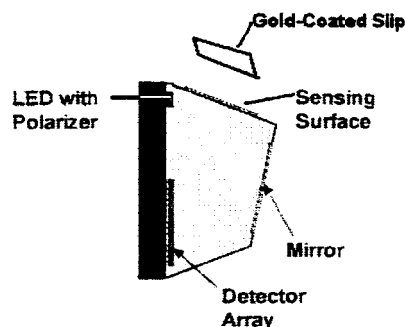
Lysozyme was driven from the anode towards the cathode (lysozyme molecules are positively charged at pH 5.98) as evidenced by a semi-circular band of crystals formed around the location of the anode, with no crystals inside this band. The crystals in the band were very small but nicely shaped. Close to the anode, the current density became higher due to our geometry, and we believe this is responsible for the poor quality of the crystals in that region. In addition, the pH in that region after electrophoresis was found to be very basic - beyond the region that lysozyme crystallizes well. Shown below is the general scheme of the crystallization.



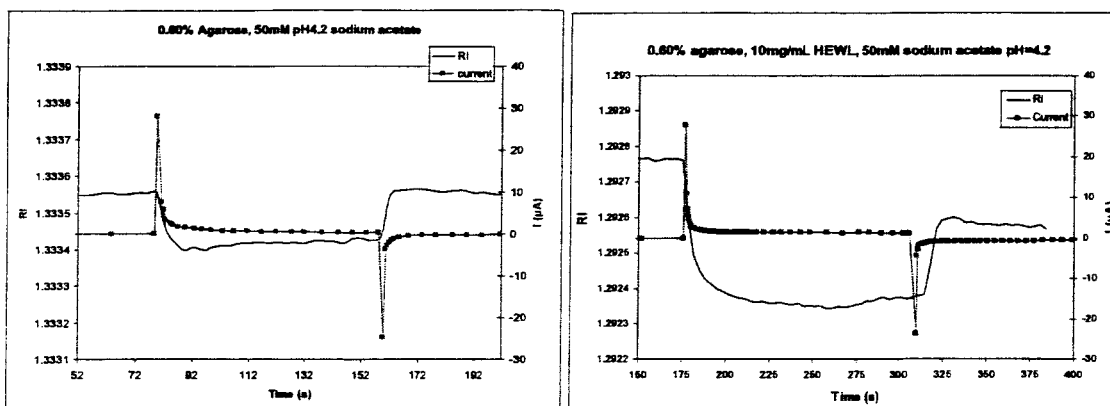
The pH variation also led to different aspect ratios of the crystals in different locations on the gel. In essence, we were able to sample a range of pH values in a single growth experiment. The poor quality of the crystals in middle of the control sample is attributed to the non-uniform distribution of precipitant, which was poured over the top of the gel.

Surface plasmon resonance studies

The introduction of commercial surface plasmon resonance (SPR) systems with disposable sensor blocks (Spreeta[®] system) has opened up many new possibilities for studies in the life sciences. For our research, we were able to use the sensor surface as one side of a capacitive cell to explore the motion of buffer ions and proteins in solution. Since the SPR only probes 2-3 nm into the liquid in which it is immersed, it has excellent sensitivity to the formation of charged dipole layers under the application of a dc field. We loaded the cell with combinations of agarose, buffer and protein, and compared their behavior under an applied voltage.



We found that due to higher mobility, buffer ion motion dominated, but that the osmotic force created by this motion could displace protein molecules near the surface. Below we show a comparison of the results of the refractive index shift (blue) and current flow (pink) through the cell for samples with and without HEWL in the cell. On the left side, the sample with no HEWL shows rapid response of the ions, causing a jump in the refractive index. Upon removal of the applied field, the refractive index recovers to its



50mM pH4.2 sodium acetate and 0.60% agarose, without (left) and with(right) 10mg/ml HEWL with 1.33kV/m field applied

original value. For the sample with protein in the solution in addition to the buffer ions, we find that there is a fast initial response, followed by a slower continued decay in the refractive index. In this latter part of the "field on" period, the HEWL continues to be displaced through the agarose, away from the sensor surface. When the field is removed, the HEWL remains displaced from the surface, as indicated by the failure of the refractive index to recover. If the bias is reversed, ions of the opposite sign move to the lower electrode, again driving the HEWL away from the sensor surface. Thus, with clever electrode design, it should be possible to corral the lysozyme, but only in volumes that would probably be too small to be of practical interest, due to the small values of the Debye length.

Protein displacements under AC fields - dielectrophoresis

Armed with the results of the dielectrophoresis modeling, we undertook a study the motion of lysozyme microcrystals in alternating electric fields. Electrodes suitable for creating highly gradient fields were developed. We first confirmed the operation of our apparatus using small dielectric spheres, as shown below.

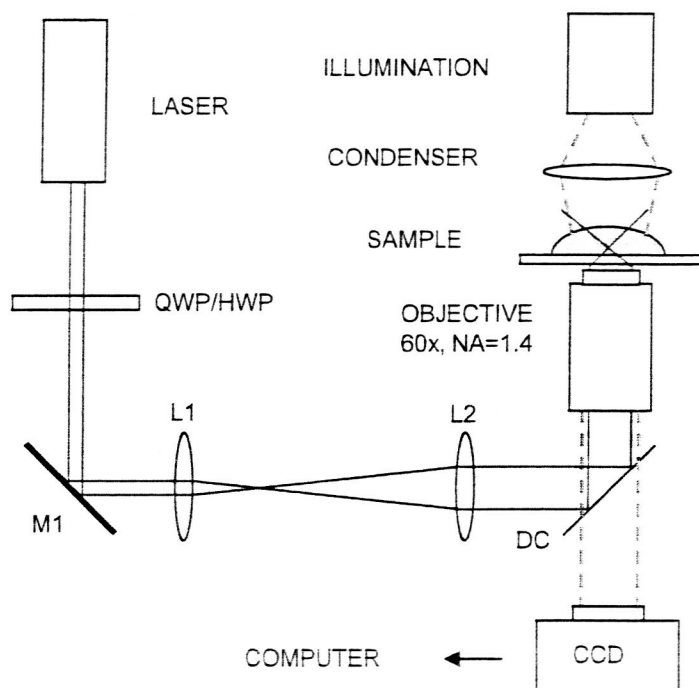


*a) before electric field
b) >100 seconds at 30V, 100KHz. Note the upper two electrodes in b) were corroded in a previous experiment, leaving them less viable than the lower 2 electrodes in b).*

These early experiments allowed us to determine the parameters (frequency, voltage, current) that the electrodes could sustain without damage. However, all attempts to move lysozyme crystals failed, despite the prediction of the model that it should be possible in the frequency range that was accessible to us. We attempted to measure the dielectric properties of the crystals, but were unsuccessful, due to impedance matching problems.

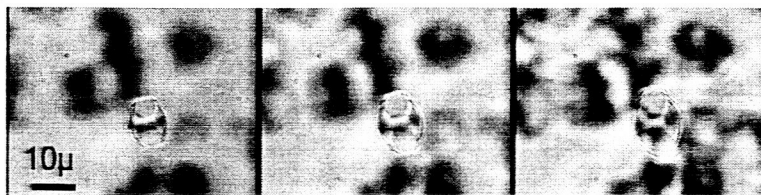
Optical frequencies – laser tweezers

We then chose to explore much higher frequencies - where any possible effects of solution DC conductivity would be irrelevant. The figure below shows the experimental setup for this work:



Setup for optical tweezers trapping and characterization of lysozyme crystal growth

With the crystals trapped at the beam waist of the tightly focused laser, we were able to observe alignment of the crystals with the polarization of the beam, and were able to induce growth while the crystal was trapped. The images below are taken from a movie of the growth of the crystal that was trapped at the beam waist. For this preliminary experiment, we added protein at high concentration to the drop where the crystal was



Growth of lysozyme seed (from pH 7, 1:4 protein:salt solution) in the tweezers after addition of protein concentrate. Total elapsed time is 1 minute 12 seconds. (567 kB movie)

trapped. Over a time of 1.2 minutes, the growth indicated below was observed. Note that the orientation of the crystal does not change; it is pinned by the polarization direction of the laser beam, despite significant concentration-gradient driven flow.

Conclusions/overall assessment:

Application of dc and low frequency electric fields to proteins during crystallization is made difficult by the large conductivity of the buffer solution in which they grow. The use of an agarose gel makes some studies possible, in the presence of a current flow. For all other cases, the electric field is restricted to a small region around the electrodes, due to shielding by the charged particles in the solution.

Even with this restriction, it is possible to perform some studies, as long as one has a probe that is sensitive to the near-electrode region. In this regard, we found that the use of a surface plasmon resonance detector, where the active surface could be biased with an electrical connection, permitted some measurable localization of the proteins.

Alternating current sources provide fewer challenges in terms of electrode corrosion; in the presence of a high frequency (10kHz+) field, the proteins can be separated using dielectrophoretic forces. Modeling of this process suggests that this may ultimately be a useful method.

Even higher frequencies (infrared light) were the most promising for localizing the growth of protein crystals. Rather than use the evanescent field of a fiber, we found that free-space propagation of a tightly focused beam permitted control of the crystal location and orientation during growth. The gradient forces of the electric field force a microcrystal seed to the focal point, and the polarization of the beam provides a force that stabilizes the orientation of the crystal. Addition of protein to the solution surrounding the crystal was seen to result in growth, and reducing the temperature should also lead to growth. A fiber-based optical tweezers arrangement could be made compatible with and x-ray diffractometer for in-situ measurements. A study of whether the growth rate at the focus of the beam is a function of laser power is the next important step.

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Ming Qi - M.S. thesis, Dartmouth College

Yi Kou – M.S. thesis, Dartmouth College

Jessica Gomez -M.S. thesis, Dartmouth College

"Buffer Effects in the Nucleation and Growth of Chicken Egg White Lysozyme" *Emily Horrell, Mark Pusey and Ursula Gibson*, American Crystallography Society meeting, ACA Annual Meeting, Buffalo, NY, May 22-27, 1999.

(<http://www.hwi.buffalo.edu/ACA/ACA-Annual/Buffalo/Buffalo.html>)

"The Effect of pH on the Growth and Aspect Ratio of Chicken Egg White Lysozyme Crystals Prepared in Different Buffers" *U. J. Gibson, E. E. Horrell, Y. Kou* (ICCBM-8), Sandestin, Florida; May 14–19 (2000) (refereed)

"Determination of crystal orientation from micrographs using a MATLAB program" *Appl. Cryst.*, *Ursula Gibson and Yi Kou*, under review

"Manipulation and growth of birefringent protein crystals in optical tweezers" *Optics Express*, *Wolfgang Singer, Halina Rubinsztein-Dunlop and Ursula Gibson*, under review

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